DOI: 10.1002/vms3.118

Original Article

Sulforaphane absorption and histone deacetylase activity following single dosing of broccoli sprout supplement in normal dogs

Kaitlin M. Curran*[1], Shay Bracha*, Carmen P. Wong[†,‡], Laura M. Beaver[†,‡], Jan F. Stevens[‡,§] and Emily Ho[†,‡,¶]

*Department of Clinical Sciences, Carlson College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, USA †School of Biological and Population Health Sciences, Oregon State University, Corvallis, Oregon, USA ‡Linus Pauling Institute, Oregon State University, Corvallis, Oregon, USA §Department of Pharmaceutical Sciences, Oregon State University, Corvallis, Oregon, USA and ¶Moore Family Center for Whole Grain Foods, Nutrition and Preventive Health, Oregon State University, Corvallis, Oregon, USA

Abstract

The role of epigenetic alterations during cancer has gained increasing attention and has resulted in a paradigm shift in our understanding of mechanisms leading to cancer susceptibility. Sulforaphane (SFN) is a naturally occurring isothiocyanate derived from the precursor glucosinolate, glucoraphanin (GFN), which is found in cruciferous vegetables such as broccoli. Sulforaphane has been shown to suppress tumour growth by several mechanisms including inhibiting histone deacetylases. The objective of this study was to provide a detailed analysis of sulforaphane absorption following a single oral dose of a broccoli sprout supplement in normal dogs. A single dose of broccoli sprout supplement (with active myrosinase) was orally administered to 10 healthy adult dogs. Blood and urine samples were collected prior to dosing, and at various time points post-dosing. Plasma total SFN metabolite levels peaked at 4 h post-consumption and were cleared by 24 h post-consumption. Urinary SFN metabolites peaked at 4 h post-consumption, and remained detectable at 24 and 48 h post-supplement consumption. A trend for decrease in histone deacetylase activity was observed at 1 h post-consumption and a significant decrease was observed at 24 h post-consumption. The data presented herein indicate that oral SFN is absorbed in dogs, SFN metabolites are detectable in plasma and urine post-dosing, and SFN and its metabolites have some effect on histone deacetylase activity following a single dose.

Keywords: dogs, histone deacetylases, neoplasms, vegetables.

Correspondence: Kaitlin M. Curran, 172 Magruder Hall, Department of Clinical Sciences, Oregon State University, Corvallis, OR, 97331, USA. E-mail: katie.curran@oregonstate.edu

Introduction

The consumption of fruits and vegetables is associated with decreased risk of cancer in people (Block *et al.* 1992; Steinmetz & Potter 1991; Riboli & Norat 2003). This inverse association is greatest when considering cruciferous vegetables (Verhoeven *et al.* 1996; Royston & Tollefsbol 2015). Sulforaphane, a naturally occurring isothiocyanate derived from its glucosinolate precursor glucoraphanin (GFN), is found in cruciferous vegetables such as broccoli. Sulforaphane has been identified as one of the critical bioactive compounds associated with the health benefits following the consumption of these vegetables, and has been shown to play a powerful role in tumour suppression (Myzak *et al.* 2005; Hsu *et al.* 2011; Clarke *et al.* 2008). In particular, targeting chromatin-modifying enzymes, such as HDAC or DNA methylation, has emerged as a potential therapeutic strategy for several cancers. Sulforaphane suppresses tumour growth and acts as a HDAC inhibitor and inhibitor of DNA methyltransferases in several animal models of cancer and in humans (Clarke *et al.*

Clinical Trial Registration:
This trial was not registered outside of the institution of origin as all study patients were internally recruited.

2011a; Myzak *et al.* 2007; Myzak *et al.* 2006). In a recently published study, women scheduled for a breast biopsy were evaluated for selective biomarkers and the presence of metabolites based after consumption of a broccoli sprout extract containing GFN (Atwell *et al.* 2015a). Patients were noted to have statistically significant increases in SFN metabolites in urine and plasma after supplementation.

For SFN to be absorbed from cruciferous vegetables, it must be released from its precursor, GFN. Most SFN is released when GFN is hydrolysed by myrosinase that cleaves a glucose and sulfate moiety from GFN to form SFN. Myrosinase enzymes are found in the plant, kept separate from glucosinolates in different cellular compartments, thereby preventing isothiocyanate formation. However, when there is damage to the plant as with chewing, cutting, etc., the myrosinase is released and forms isothiocyanates (Atwell *et al.* 2015b). The gut microbiota also contains a small amount of enzymes with myrosinase-like activity. When myrosinase is inactivated, SFN absorption is significantly reduced with broccoli sprout extract supplementation compared to fresh broccoli sprouts in healthy human adults (Clarke *et al.* 2011b; Clarke *et al.* 2011c).

In veterinary medicine, SFN has been investigated, *in vitro*, alone and with doxorubicin against osteosarcoma cell lines (Rizzo *et al.* 2017). The results indicate that SFN significantly decreased cell invasion and downregulation of focal adhesion kinase signalling. However, to date, there have been no studies evaluating the consumption of SFN *in vivo*, in normal dogs or dogs with cancer.

The goal of this study was to provide an analysis of SFN absorption and urinary excretion following a single oral dosing of a broccoli sprout supplement (with active myrosinase; BroccoMax™, Jarrow Formulas®, Los Angeles, CA, USA) in normal dogs. Additionally, we sought to evaluate HDAC inhibition in peripheral blood cells after SFN dosing. Our hypothesis was that dogs would have similar absorption and urinary excretion data compared to humans. Our findings provide baseline information for the design of future SFN supplementation trials in dogs with and without disease.

Materials and methods

Patient selection

Ten healthy dogs over 1 year of age were recruited. This study was conducted with client-owned dogs. Inclusion criteria included patient weight between 25 and 36 kg, adequate organ function assessed by complete blood count, serum biochemistry and urinalysis, and normal performance status and activity level. Exclusion criteria included the presence of malignancy or other serious systemic disorder (i.e. immune-mediated disease, renal failure), consumption of broccoli, garlic or onions within 2 days of the study start, or dogs on homoeopathic or alternative therapies. All participating dog owners signed informed client consent forms. The study protocol (no. 4790) was approved by the Institutional Animal Care and Use Committee at Oregon State University (OSU) and carried out in accordance with the relevant guidelines and regulations.

Dietary intervention

All dogs were fed commercial kibble as per their regular at-home husbandry starting 3 days prior to the study start. Owners were instructed to avoid all treats during this time. On Day 0 of the study, dogs were orally administered three capsules of a broccoli sprout supplement (BroccoMax™) to achieve 90 mg total of GFN (per Jarrow Formulas®, 30 mg of GFN yields 8 mg SFN *in vitro*). The GFN dose used was based on estimated weight equivalent dose in people (Atwell *et al.* 2015a). All dogs swallowed the supplement without chewing, and received their morning meal immediately following the broccoli sprout supplement dosing (BroccoMax™). Dogs were followed up for 48 hours post-dosing and owners were asked to identify any adverse events noted at home. Adverse events were graded based on the Veterinary Comparative Oncology Group common terminology criteria for AEs (Veterinary Cooperative Oncology Group, 2016).

Sample collection

Blood and urine samples were collected prior to dosing with glucoraphanin on the morning of Day 0 (baseline), and at various time points post-dosing.

Urine samples were obtained by voiding at baseline, 4, 24 and 48 h post-dosing. Samples were divided and one half of the sample was acidified with trifluoroacetic acid to a final concentration of 10% v/v. The remaining half of the sample was left unacidified. Whole blood samples were collected in vacutainers containing EDTA (EDTA vacutainers, Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) at baseline, 1, 2, 4, 8, 24 and 48 h post-dosing. For plasma collection, samples were centrifuged at 600 rcf for 10 min, and plasma was removed. A quantity of 500 μL of plasma samples were immediately acidified with trifluoroacetic acid to a final concentration of 10% v/v. The remaining plasma samples were left unacidified. For PBMC isolation, whole blood samples were collected at baseline, 1, 2 and 24 h post-dosing, and processed as previously described (Clarke *et al.* 2011b) using cell separation media (Histopaque®-1077, Sigma-Aldrich Co. LLC., St. Louis, MO, USA). PBMC protein lysates were obtained by resuspending PBMC pellets in IP lysis buffer containing protease inhibitors. PBMC protein concentrations were determined using a detergent compatible protein assay (DC^{TM} protein assay, Bio-Rad, Hercules, CA, USA). All samples were stored at −80°C after processing.

Sample analysis

Sulforaphane metabolite quantification

Plasma and urine samples were processed and analysed as previously described (Atwell *et al.* 2015b). Briefly, acidified plasma and urine samples were centrifuged for 5 min at 4°C, 12,000*g* to remove protein precipitates. Supernatants were collected and filtered using centrifuge tube filter columns (0.22-μm Spin-X® filter columns, VWR International, Radnor, PA, USA). Filtered urine samples were further diluted twofold in 0.1% formic acid (v/v). Sulforaphane metabolites in filtered samples were analysed using a liquid chromatography–mass spectrometry instrument (MDS Sciex 4000 QTRAP LC-MS/MS, Applied Biosystems, Foster City, CA, USA) at the OSU Mass Spectrometry Center. The following precursor and product ions were used to detect SFN and its metabolites: SFN (178 > 114), SFN-GSH (485 > 179), SFN-Cys (299 > 114), SFN-NAC (341.1 > 114) and SFN-CG (356 > 114). Quantification was determined against known standards using 8-point linear standard curves (r^2=0.99).

Urine creatinine quantification

For urine samples, creatinine was determined using the standard Jaffe's reaction. Urinary SFN metabolites were normalized to urinary creatinine levels.

PBMC HDAC activity

Histone deacetylases activity was measured from PBMC lysates as previously described (Myzak *et al.* 2004). Samples were analysed using the HDAC inhibition assay at the Cancer Prevention and Intervention Core Labs at the Linus Pauling Institute at OSU.

Statistical analyses

Statistical analyses were performed using commercially available software (GraphPad Prism Version 5.02, GraphPad Software, Inc., La Jolla, CA, USA). All data were reported as mean ± SEM. *P* values were determined using one-way ANOVA. When there was a significant main effect, Dunnett's *post hoc* tests were used to determine the differences between the means at different time points post-SFN supplement consumption compared to 0 h baseline. Statistical significance was defined as $P \leq 0.05$.

Results

Patient characteristics

Ten dogs met the inclusion criteria and successfully completed the study. Represented dog breeds included Labrador (n = 5), goldendoodle (n = 3), Australian Shepherd (n = 1) and mixed (n = 1). The median patient age was 6.6 years (range: 1.1–11.0), and median patient weight was 28.3 kg (range: 20.2–35.4). All dogs were fed a dry commercial dog food

and were on no medications apart from glucosamine and chondroitin supplements.

All dogs were dosed with a broccoli sprout supplement (BroccoMax™) and successfully completed blood and urine sampling over the following 48 h. No AEs attributed to the broccoli sprout supplement were noted by dog owners or clinicians during the 48-h study period. However, one dog had grade II vomiting noted 72-h following broccoli sprout supplement (BroccoMax™) dosing. This AE was due to foreign body ingestion.

Sulforaphane metabolites were detected in plasma and urine samples post-broccoli sprout supplement consumption

Sulforaphane metabolites were detected in plasma samples in 9 out of 10 dogs following broccoli sprout supplement (BroccoMax™) consumption. Plasma total SFN metabolite levels peaked at 4 h post-consumption, and were cleared by 24 h post-consumption (Fig. 1a). SFN-GSH was the major SFN metabolite detected in plasma (Fig. 1b and Table 1). In urine samples, SFN metabolites were detected for all 10 dogs following broccoli sprout supplement consumption. Urinary SFN metabolites peaked at 4 h post-consumption and remained detectable at 24 and 48 h post-supplement consumption (Fig. 2a). One dog that did not have detectable plasma SFN metabolites had detectable urinary SFN metabolites, but the peak urinary metabolites was delayed until 24 h. Sulforaphane and SFN-Cys were the two major SFN metabolites detected in urine samples (Fig. 2b and Table 2).

Histone deacetylase activity

Histone deacetylase activity was determined in PBMC at 0, 1, 2 and 24 h post-SFN supplement consumption (Fig. 3). A trend for decrease in HDAC activity was observed at 1 h post-consumption (11.3% decrease vs. baseline) and a significant decrease was observed at 24 h post-consumption (24.4% decrease vs. baseline). One dog was excluded from the analysis due to haemolysis in the PBMC sample.

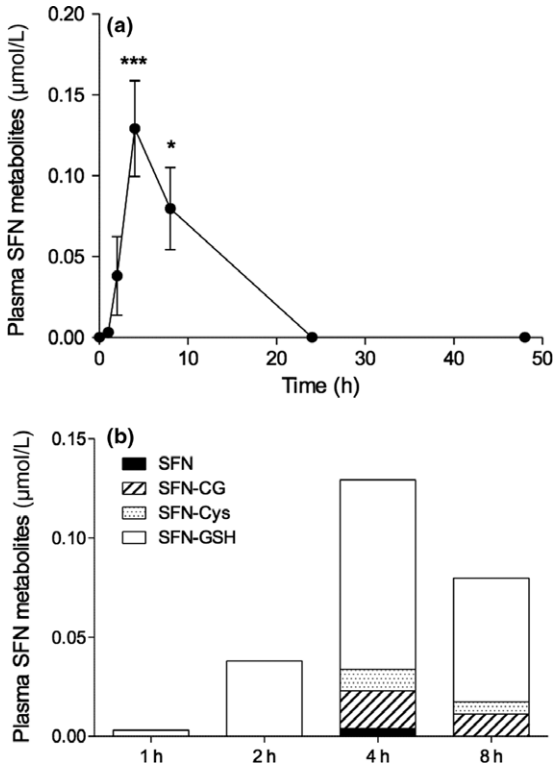

Fig. 1. Plasma sulforaphane (SFN) metabolite levels following SFN supplement consumption. (a) Total SFN levels (all metabolites) at 0 h (baseline), 1, 2, 4, 8, 24 and 48 h post-consumption (mean ± SEM). (b) Distribution of individual SFN metabolites (SFN, SFN-CG, SFN-Cys and SFN-GSH) in plasma samples at 1, 2, 4 and 8 h. SFN metabolites were not detected at 0, 24 and 48 h. ***$P < 0.001$, *$P < 0.05$.

Discussion

This study is the first to evaluate the administration of a broccoli sprout supplement in normal dogs. Based on this study, SFN and SFN metabolites had peak plasma and urine concentration at 2 and 4 hrs, respectively. In plasma, SFN and metabolite concentrations reached low levels 24 hours after consumption of a single dose, indicating rapid clearance from the plasma. Sulforaphane metabolites in urine reached low levels 48 h after consumption. These dynamics are similar to those found in humans, with peak SFN and metabolite concentrations within several hours of dosing and clearance from the plasma within 24 h (Atwell *et al.* 2015b; Clarke *et al.* 2011b). Specifically, in our patient population, micromolar

Table 1. Plasma sulforaphane (SFN) metabolites summary.

Plasma SFN metabolites mean (SE)				
	1 h	2 h	4 h	8 h
SFN	ND	ND	0.004 (0.003)*	ND
SFN-CG	ND	ND	0.019 (0.007)*	0.011 (0.006)
SFN-Cys	ND	ND	0.011 (0.007)	0.006 (0.006)
SFN-GSH	0.003 (0.003)	0.038 (0.024)	0.095 (0.019)*	0.062 (0.014)*
SFN-NAC	ND	ND	ND	ND

Plasma SFN metabolite concentrations are shown in micromolar (μmol/L) concentrations. ND, not detected.*$P < 0.05$ compared to 0 h baseline.

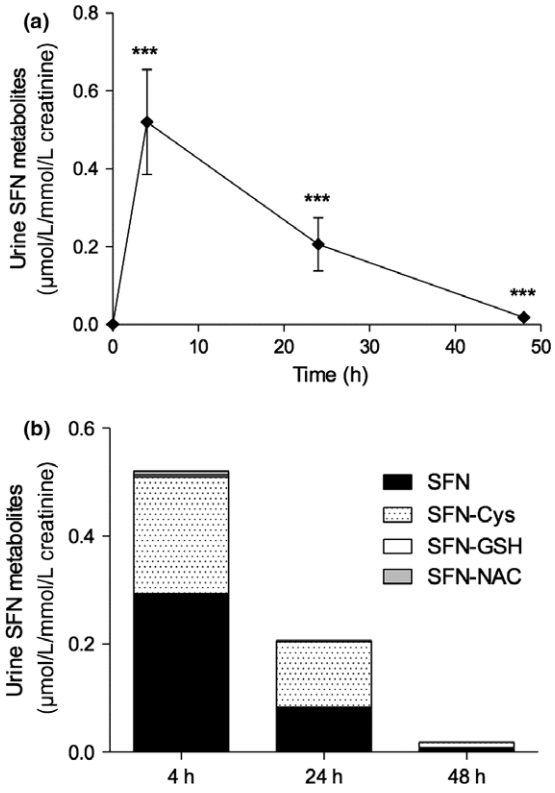

Fig. 2. Urine sulforaphane (SFN) metabolite levels following SFN supplement consumption. (a) Total SFN levels (all metabolites) at 0 h (baseline), 4, 24 and 48 h post-consumption (mean ± SEM). (b) Distribution of individual SFN metabolites (SFN, SFN-Cys and SFN-NAC) in urine samples at 4, 24 and 48 h. SFN metabolites were not detected at 0 h baseline. ***$P < 0.001$.

Table 2. Urinary sulforaphane (SFN) metabolites summary.

Urinary SFN metabolites mean (SE)			
	4 h	24 h	48 h
SFN	0.294 (0.087)*	0.083 (0.001)	0.008 (0.001)
SFN-CG	ND	ND	ND
SFN-Cys	0.216 (0.072)*	0.121 (0.035)	0.010 (0.002)
SFN-GSH	0.004 (0.001)*	ND	ND
SFN-NAC	0.007 (0.002)*	ND	ND

Urinary SFN metabolite concentrations are shown micromolar (μmol/L) concentrations of urinary SFN metabolite/millimolar (mmol/L) concentrations of urinary creatinine. ND: not detected.*$P < 0.05$ compared to 0 h baseline.

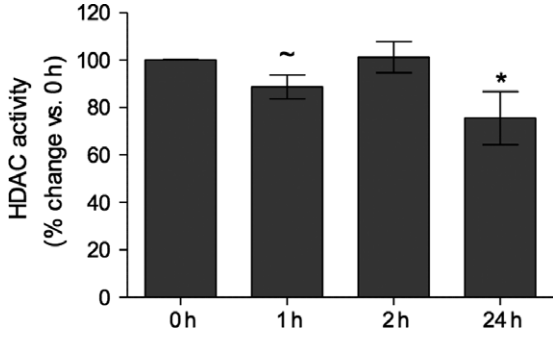

Fig. 3. Histone deacetylase (HDAC) activities in PBMC following sulforaphane supplement consumption. HDAC activity was determined in peripheral blood mononuclear cells at 0 h (baseline), 1, 2 and 24 h post-consumption. Data represent percentage change in HDAC activity compared to baseline (mean ± SEM). *$P < 0.05$, ~$P < 0.08$.

plasma concentration of SFN and SFN metabolites peaked at 4 h (range at peak of 0.004–0.095 μmol/L) which is similar to findings in humans after a single dose of broccoli sprout extract (peak noted at 3 h; range of SFN metabolites 0.1–0.2 μmol/L) (Atwell *et al.* 2015b).

This study provides the first evidence that SFN is bioavailable following broccoli supplement

consumption in canines, with limited adverse events. This work lays the groundwork for the clinical study of SFN supplementation in disease prevention and/or treatment in veterinary practice. In particular, this work will serve as the basis for the future studies examining the impact of longer term SFN supplementation, and its impact in dogs with and without cancer.

Sulforaphane may help inhibit cancer formation and progression by multiple epigenetic and non-epigenetic mechanisms, including inhibiting HDAC activity (Clarke *et al.* 2008; Ellis *et al.* 2009). The peripheral blood mononuclear cell HDAC activity identified in this study population was similar to what has previously been reported in normal human subjects supplemented with SFN (Atwell *et al.* 2015b). Furthermore, our study population had a decrease in HDAC activity by 24.4% from baseline. By comparison, women scheduled for breast biopsy following SFN supplementation had a decrease of 18% (Atwell *et al.* 2015a). Histone deacetylase activity may decrease more significantly in subjects with abnormal or transformed cells as with cancer (Clarke *et al.* 2011a; Weichert 2009). However, with routine dosing, additional reduction in HDAC activity may be noted in future work in dogs.

The limitations of this study include a small sample size, variation in patient weight and age, and variation in patient diet. However, some level of patient variation is more similar to the typical dog population presenting to a veterinary clinic. Additionally, an individual dog's diet may have affected SFN absorption. However, all dogs consumed their typical commercial dry dog food breakfast following SFN dosing. The dog foods provided did not contain SFN or compounds known to influence SFN absorption. All patients swallowed the broccoli supplement capsules whole and were not able to chew them prior to swallowing. This may have decreased the release of myrosinase, but would have been uniform across patients.

The role of epigenetic alterations during cancer has gained increasing attention and has resulted in a paradigm shift in our understanding of mechanisms leading to cancer susceptibility. The impact of dietary HDAC inhibitors, such as SFN and its metabolites, has only begun in canine patients. The data presented herein indicate that oral SFN is absorbed by dogs, SFN metabolites are present in plasma post-dosing, and SFN and its metabolites have some effect on HDAC activity following a single dose. Future clinical studies should include work to determine safety and early mechanistic targets, as well as randomized, placebo-controlled clinical trials using SFN supplementation to potentially prevent or slow cancer development in canine patients.

Acknowledgements

The authors acknowledge the Oregon State University Mass Spectrometry Core for contribution to this project, and thank Drs. Leeper and Parachini-Winter for technical assistance. This work was supported in part by an internal grant at the Carlson College of Veterinary Medicine, Oregon State University. 4000 QTRAP mass spectrometry was supported by the National Institutes of Health, NIH grant number S10RR022589.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Ethical Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

Contributions

Design of the experiments: KM Curran, S Bracha, C Wong and E Ho. Sample collection: KM Curran and S Bracha. Experiments: C Wong, LM Beaver, JF Stevens and E Ho. Manuscript draft and revision: KM Curran, S Bracha, C Wong, LM Beaver, JF Stevens, E Ho.

References

Atwell L.L., Zhang Z., Mori M., Farris P.E., Vetto J.T., Naik A.M. *et al.* (2015a) Sulforaphane bioavailability

and chemopreventive activity in women scheduled for breast biopsy. *Cancer Prevention Research* **2015**, 1184–1191.

Atwell L.L., Hsu A., Wong C.P., Stevens J.F., Bella D., Yu T.W. *et al.* (2015b) Absorption and chemopreventive targets of sulforaphane in humans following consumption of broccoli sprouts or a myrosinase-treated broccoli sprout extract. *Molecular Nutrition & Food Research* **59**, 424–433.

Block G., Patterson B. & Subar A. (1992) Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutrition and Cancer* **18**, 1–29.

Clarke J.D., Dashwood R.H. & Ho E. (2008) Multi-targeted prevention of cancer by sulforaphane. *Cancer Letters* **269**, 291–304.

Clarke J.D., Hsu A., Yu Z., Dashwood R.H. & Ho E. (2011a) Differential effects of sulforaphane on histone deacetylases, cell cycle arrest and apoptosis in normal prostate cells versus hyperplastic and cancerous prostate cells. *Molecular Nutrition & Food Research* **55**, 999–1009.

Clarke J.D., Riedl K., Bella D., Schwartz S.J., Stevens J.F. & Ho E. (2011b) Comparison of isothiocyanate metabolite levels and histone deacetylase activity in human subjects consuming broccoli sprouts or broccoli supplement. *Journal of Agricultural and Food Chemistry* **59**, 10955–10963.

Clarke J.D., Hsu A., Riedl K., Bella D., Schwartz S.J., Stevens J.F. & Ho E. (2011c) Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmacological Research* **64**, 456–463.

Ellis L., Atadja P.W. & Johnstone R.W. (2009) Epigenetics in cancer: targeting chromatin modifications. *Molecular Cancer Therapeutics* **8**, 1409–1420.

Hsu A., Wong C.P., Yu Z., Williams D.E., Dashwood R.H. & Ho E. (2011) Promoter de-methylation of cyclin D2 by sulforaphane in prostate cancer cells. *Clinical Epigenetics* **3**, 3.

Myzak M.C., Karplus P.A., Chung F.L. & Dashwood R.H. (2004) A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. *Cancer Research* **64**, 5767–5774.

Myzak M.C., Hardin K., Wang R., Dashwood R.H. & Ho E. (2005) Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. *Carcinogenesis* **27**, 811–819.

Myzak M.C., Dashwood W.M., Orner G.A., Ho E. & Dashwood R.H. (2006) Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc min mice. *The FASEB Journal* **20**, 506–508.

Myzak M.C., Tong P., Dashwood W.M., Dashwood R.H. & Ho E. (2007) Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *Experimental Biology and Medicine* **232**, 227–234.

Riboli E. & Norat T. (2003) Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *American Journal of Clinical Nutrition* **78**, 559S–569S.

Rizzo V.L., Levine C.B. & Wakshlag J.J. (2017) The effects of sulforaphane on canine osteosarcoma proliferation and invasion. *Veterinary and Comparative Oncology* **15**, 718.

Royston K.J. & Tollefsbol T.O. (2015) The epigenetic impact of cruciferous vegetables on cancer prevention. *Current Environmental Health Reports* **1**, 46–51.

Steinmetz K.A. & Potter J.D. (1991) Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes & Control* **2**, 427–442.

Verhoeven D.T., Goldbohm R.A., van Poppel G., Verhagen H. & van den Brandt P.A. (1996) Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiology and Prevention Biomarkers* **5**(9), 733–748.

Veterinary cooperative oncology group (2016) common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Veterinary and Comparative Oncology* **14**, 417.

Weichert W. (2009) HDAC expression and clinical prognosis in human malignancies. *Cancer Letters* **280**, 168–176.